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13. SUPPLEMENTARY NOTES

14. ABSTRACT Duchenne muscular dystrophy (DMD) is a life threatening disease affecting all muscles in the body. An important therapeutic goal of DMD gene therapy is to deliver a therapeutic gene to all muscles in the body. The overarching goal of this project is to achieve systemic AAV micro-dystrophin gene therapy in young adult affected dogs. In the last funding period, we proposed to incorporate the CpG-free feature to our vector to further diminish the untoward immune response. In this funding period, we demonstrated that elimination of CpG from AAV ITR does not affect therapeutic efficacy but it reduces packaging efficiency. We also showed that CpG-free microgenes are functional. However, removal of hinge3 resulted in better protection against eccentric contraction. We further showed that the CK8 promoter is highly effective in driving muscle-specific expression. Based on these results, we develop a novel XP49 vector. In this vector, a CpG-free codon-optimized, hinge3-deleted human microgene is expressed from the CK8 promoter. We will move forward with this vector for dog studies and future human trials. In the last funding period, we showed that systemic delivery of a canine micro-dystrophin AAV vector is safe in young adult affected dogs. We now further extended this result and demonstrated robust expression for 12 months. Importantly, we observed amelioration of muscle pathology and improvement of muscle force. In addition, we have developed a novel noninvasive assay to evaluate whole body mobility in dogs. Finally, we published three review papers on the current status of AAV DMD gene therapy.

15. SUBJECT TERMS
Duchenne muscular dystrophy, DMD, dystrophin, micro-dystrophin, adeno-associated virus, AAV, muscle, gene therapy, systemic gene delivery, canine model

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1. Introduction

Duchenne muscular dystrophy (DMD) is a life threatening disease affecting approximately one in 5,000 newborn boys. It is caused by dystrophin deficiency. Adeno-associated virus (AAV)-mediated micro-dystrophin gene therapy has resulted in unprecedented successful in mouse models of DMD. We propose to develop systemic AAV micro-dystrophin gene therapy in the canine model.

2. Keywords

Duchenne muscular dystrophy, DMD, dystrophin, micro-dystrophin, adeno-associated virus, AAV, muscle, gene therapy, systemic gene delivery, canine model

3. Accomplishments

Major goal. We have two specific aims. Our first aim is to design and validate a low immunogenic ΔR2-15/ΔR18-19/ΔR20-23/ΔC microgene AAV vector. Our second aim is to test systemic gene therapy in young adult DMD dogs. In the last two years, we have successfully engineered and validated muscle-specific low-immunogenic AAV micro-dystrophin vector for testing in the canine DMD model. We have also successfully achieved systemic micro-dystrophin delivery in affected dogs. In this last funding period, we have focused on (1) monitoring the dogs that have received systemic AAV microgene therapy to see if we can achieve long-term micro-dystrophin expression; (2) AAV vector production and dosing of the new XP49 vector described in the last progress report; (3) development of new physiological assays to study sympatholysis and functional ischemia in dogs in order to demonstrate the unique therapeutic benefits of nNOS-binding domain containing micro-dystrophin.

Accomplishment 1. *Monitoring the dogs that have received systemic AAV therapy to see if we can achieve long-term persistent micro-dystrophin expression.*

In the last report, we showed micro-dystrophin expression for 12 months after a single intravenous injection AAV microgene delivery. We also showed blood profiles up to 48 weeks post-injection demonstrating lack of toxic or side effect. Below we show biopsy data up to 24 months after injection **(Figure 1)** and blood profiles up to 90 weeks after injection **(Figure 2)**. Our data suggest that a one-time therapy in young adult dystrophic dogs resulted in persistent micro-dystrophin expression for two years and importantly, the blood profiles were all within the expected ranges and there is no adverse reaction.

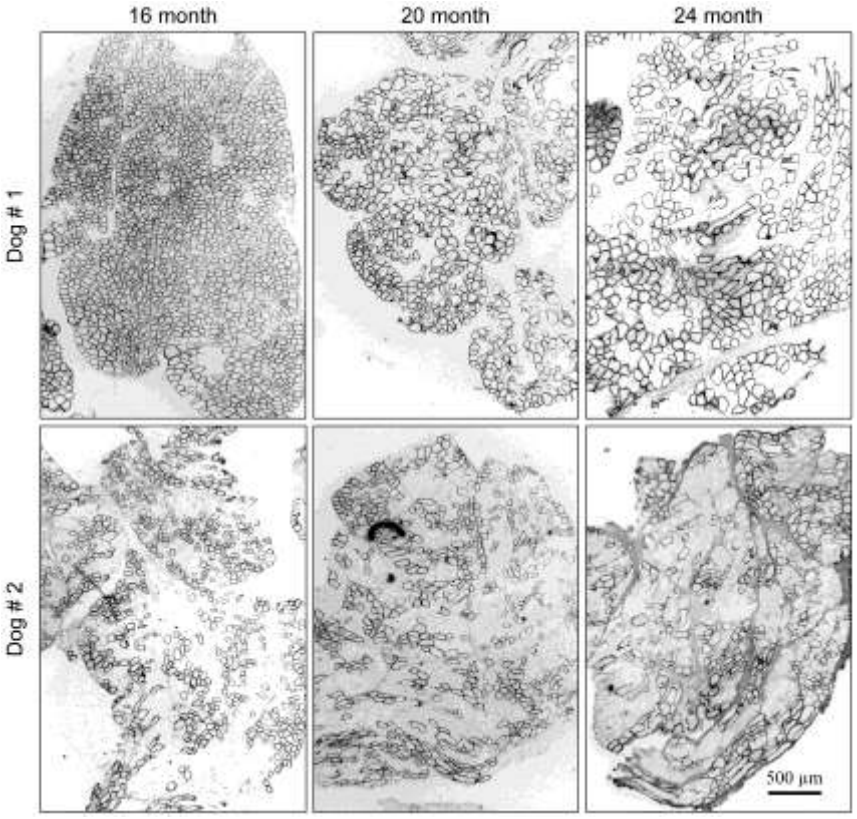

Figure 1. A single intravenous injection of AAV micro-dystrophin vector resulted in persistent AAV transduction for up to 24 months.

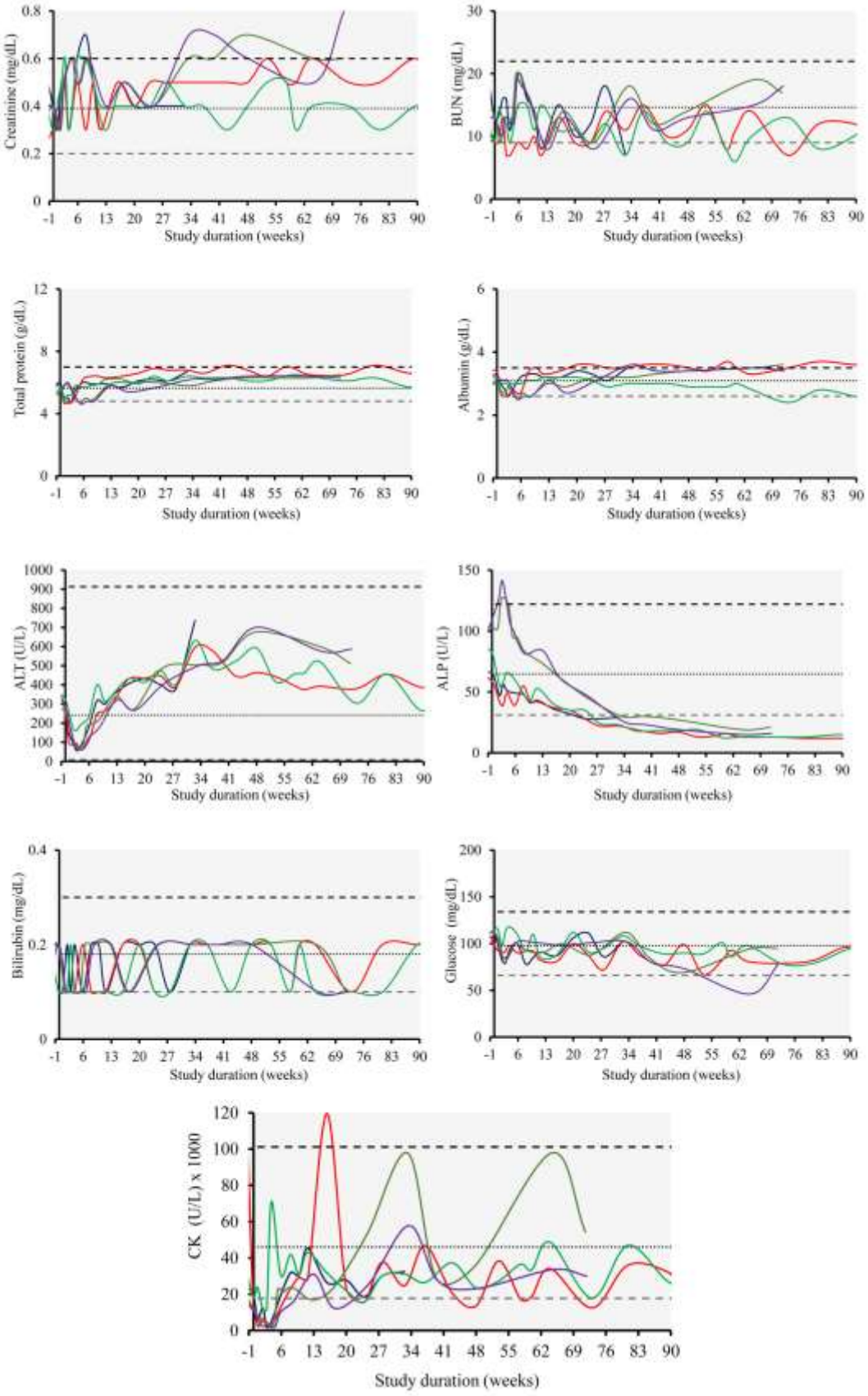

Figure 2. A single intravenous injection of AAV micro-dystrophin vector did not alter blood profile for up to 90 weeks. Each color represents a different dog.

Accomplishment 2. *Production and dosing of XP49, an updated AAV micro-dystrophin vector.*

In last progress report, we reported successful engineering of XP49, an updated AAV micro-dystrophin vector **(Figure 3)**. This vector has a number of unique properties that should improve therapeutic benefits. In particular, (1) it uses the latest muscle specific promoter, the CK8 promoter to drive micro-dystrophin expression; (2) it contains the R16/17 nNOS-binding domain to restore nNOS homeostasis; (3) it has four in-phase spectrin-like repeats; (4) the deleterious hinge 2 is removed from the microgene construct; (5) it includes the syntrophin/dystrobrevin-binding site; (6) it carries the Dys-2 epitope to allow us to perform biochemical assays to confirm the full-length micro-dystrophin expression; (7) all the CpG motif has been removed from the microgene to minimize immune response; (8) it carries the miR142-3p target site to reduce antigen presentation in macrophages; (9) it is codon-optimized; (10) the cis-packaging plasmid has an enlarged backbone to prevent untowards packaging of the backbone in AAV production, and (11) it is a human micro-dystrophin gene. In all the published and ongoing AAV microgene therapy studies in the canine model, investigators have used the canine version microgene. The canine gene cannot be used in human directly. Here, we switched to the human version microgene, so that the microgene AAV vector tested in affected dogs can be directly used in human patients in the future.

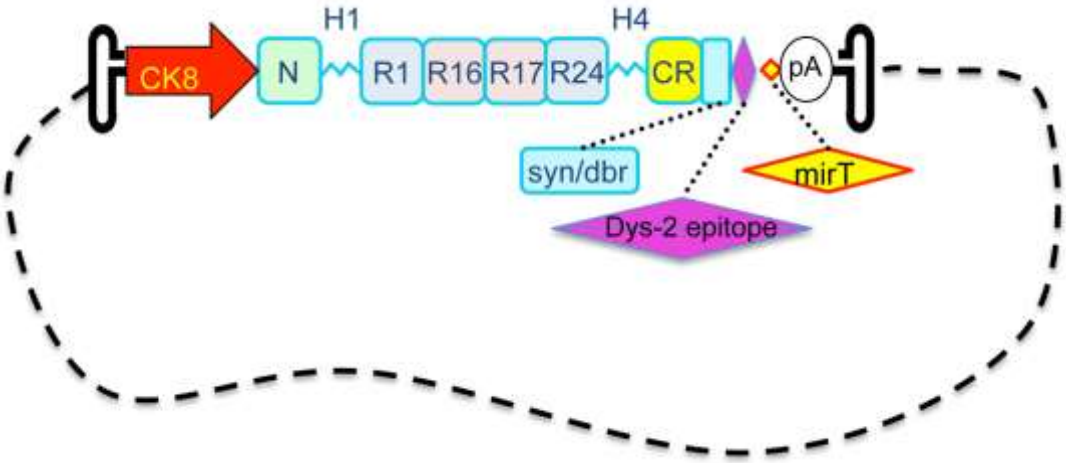

Figure 3. Schematic outline of the XP49 vector. The total size of the AAV vector genome is 4844 bp and the total size of the backbone is 6.8 kb.

We have now produced sufficient AAV-8 XP49 vector and delivered them to 6 affected dogs. All the dogs tolerated injection well. We did not see any acute toxicity.

Accomplishment 3. *Development of new physiological assays to study sympatholysis and functional ischemia in dogs.*

Functional ischemia is an important pathogenic factor in the initiation and progression of muscle disease in Duchenne muscular dystrophy. This is mainly due to failure to anchor nNOS to the sarcolemma. Unfortunately, the first generation micro-dystrophin gene cannot restore nNOS to the sarcolemma. We discovered R16/17 as the critical nNOS-binding domain in dystrophin. Hence, we engineered the second-generation R16/17-containing micro-dystrophin gene. We showed in mouse models that R16/17-containing microgene can effectively counteract functional ischemia and improve therapeutic efficacy. The canine DMD model has been established since 1988 and DMD dogs have been used in numerous studies to evaluate pharmacological and genetic therapies for DMD. Yet, there

has been no study on functional ischemia in DMD dogs. To fill this knowledge gap, we developed a novel assay to quantify limb muscle blood flow in resting and contracting dog muscle during this funding period. This assay will allow us to test whether R16/17-containing micro-dystrophin vector can effectively prevent functional ischemia in the canine DMD model.

The protocol was developed on our previously published in situ ECU muscle force assay **(Figure 4)**. A similar set-up was used except the placement of a flow probe inside the brachial arterial for quantifying blood flow changes at rest and during contraction in the absence and presence of NE administration. **Figure 5** illustrates the step-by-step protocol.

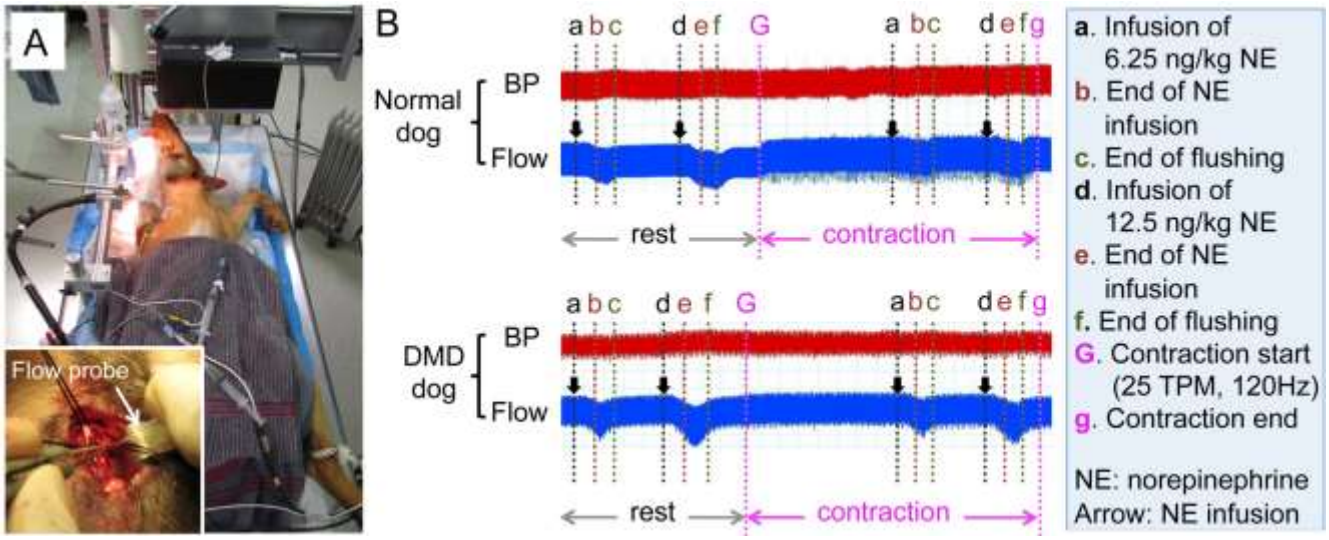

Figure 4. Development of a novel physiological assay to study functional ischemia in dogs. **A**, Dog hemodynamic assay set-up. **B**, Representative tracing from a normal (top panel) and an affected (bottom panel) dogs. During contraction, NE-induced reduction of the blood flow is blunted in normal dogs but not in affected dogs.

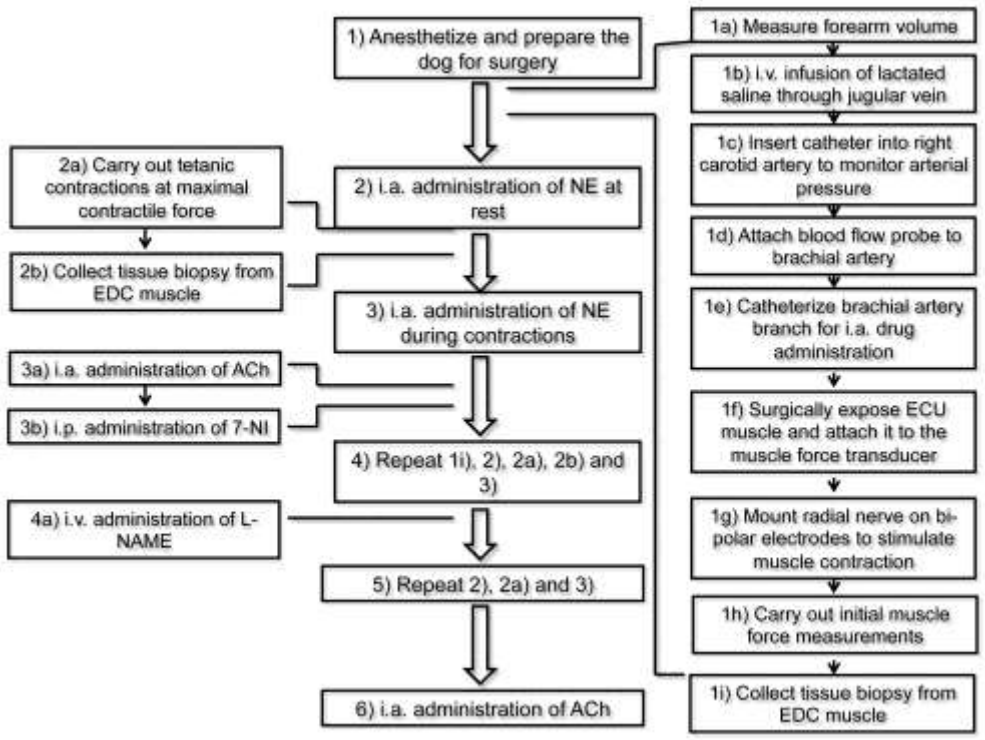

Figure 5. Step-by-step illustration of the protocol used for studying the dog muscle perfusion at rest and during contraction. Following surgery preparation, we first quantified the blood flow at rest and contraction in the absence of non-epinephrine (NE). Then we quantified blood flow in the presence of NE and various chemicals.

Normal and DMD dogs had a similar mean arterial pressure (MAP) at the baseline **(Table 1)**. The baseline mean artery blood flow in the brachial artery (MABF) of DMD dogs only reached ~50% of that of normal dogs **(Table 1)**. Nevertheless, the forearm volume normalized mean vascular conductance (MVC) was also similar between normal and DMD dogs at the baseline. Forelimb contraction at the maximal contractile force significantly increased MABF and MVC in both normal and DMD dogs **(Table 1)**.

Table 1. Blood flow hemodynamic profiles

Drug condition	Muscle contractile status	Group	MAP (Hgmm)	MABF (mL/min)	MVC (mL/min/Hgmm/100mL
Baseline					
	Rest				
		Normal	70.82 ± 3.27	102.00 ± 18.78	0.44 ± 0.06
		DMD	70.29 ± 3.95	57.93 ± 7.56[a]	0.42 ± 0.04
	Contraction				
		Normal	69.50 ± 4.06	123.00 ± 24.97[b]	0.52 ± 0.07[b]
		DMD	69.93 ± 3.33	66.43 ± 7.70[a,b]	0.50 ± 0.05[b]
After 7-NI					
	Rest				
		Normal	70.10 ± 3.33	100.60 ± 16.12	0.46 ± 0.06
		DMD	67.14 ± 3.58	51.00 ± 5.43[a]	0.41 ± 0.04
	Contraction				
		Normal	69.00 ± 3.19	126.56 ± 22.21[b]	0.56 ± 0.06[b]
		DMD	66.86 ± 3.25	62.57 ± 6.07[a,b]	0.51 ± 0.05[b]
After L-NAME					
	Rest				
		Normal	84.20 ± 3.83[c]	132.10 ± 19.43[c]	0.50 ± 0.06
		DMD	80.21 ± 3.49[c]	62.79 ± 6.40[a,c]	0.42 ± 0.04
	Contraction				
		Normal	84.90 ± 3.70[c]	152.40 ± 21.90[b,c]	0.58 ± 0.07[b]
		DMD	81.93 ± 3.38[c]	75.07 ± 6.81[a,b,c]	0.50 ± 0.05[b]

Abbreviations: MAP, mean arterial pressure; MABF, mean arterial blood flow; MVC, mean vascular conductance; DMD, Duchenne muscular dystrophy.

a, Significantly different from that of normal.

b, Significantly different from that of at rest.

c, Significantly different from that of at baseline and after 7-NI.

To assess sympatholysis, we administrated NE to induce sympathetic vasoconstriction at rest and during muscle contraction **(Figure 6, Tables 2 and 3)**. In normal dogs, NE administration resulted in 63.76 ± 5.62% reduction of MVC at rest but only 29.46 ± 4.68% during contraction ($P < 0.05$, **Figure 6C, Table 2**). Sympatholysis efficiency reached 56.34 ± 5.07% ($P < 0.05$, rest vs contraction; **Figure 6E, Table 2**). In DMD dogs, NE administration resulted in 76.89 ± 2.51% reduction of MVC at rest and 56.82 ± 3.15% reduction during contraction ($P < 0.05$, rest vs contraction; **Figure 6D, Table 2**). Interestingly, the sympatholytic efficiency between rest and contraction also reached statistical significance for DMD dogs. Nevertheless, the sympatholytic efficiency in DMD dogs (25.72 ± 3.77%) was significantly lower than that of normal dogs ($P < 0.05$, normal vs DMD; **Figure 6E, Table 2**).

Our protocol reliably portrayed a hemodynamic profile consistent with classic reflex sympathetic vasoconstriction during exercise. In normal dogs, administration of NE significantly reduced the artery conductance in resting muscle **(Figure 6A and C, Table 3)**. This vasoconstriction effect is significantly blunted in contracting muscle **(Figure 6A and C, Table 3)**. Establishment of this protocol opens the door to study the mechanisms of sympatholysis and to test therapeutic interventions aimed at improving sympatholysis in large animal models.

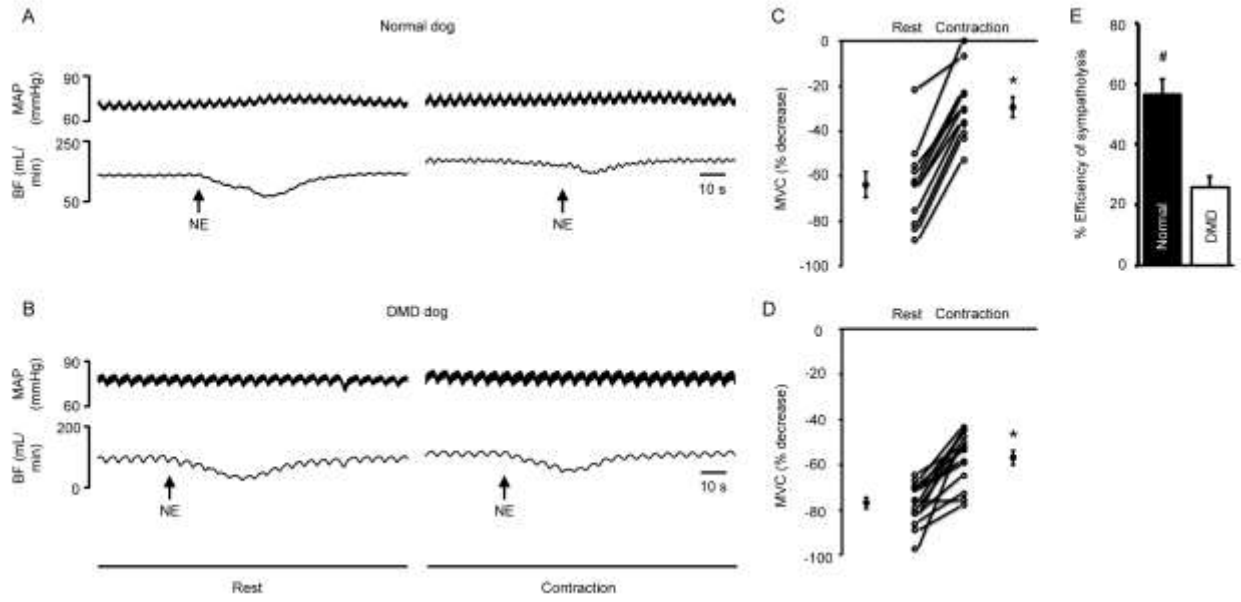

Figure 6. Attenuation of sympathetic vasoconstriction in contracting skeletal muscle is impaired in DMD dogs. (A and B) Representative tracing from a normal and a DMD dog. MAP, mean arterial pressure; BF, blood flow; NE, norepinephrine (NE). **(C and D)** MVC (mean vascular conductance) changes in normal (n = 11) and DMD dogs (n = 14). **(E)** Efficiency of functional sympatholysis in normal and DMD dogs. * significantly different from rest. # significantly different from DMD.

Table 2. Change in hemodynamic responses to NE induced sympathetic vasoconstriction

Drug condition	Muscle contractile status	Group	Δ MAP (Hgmm)	Δ MABF (mL/min)	Δ MVC
Baseline					
	Rest				
		Normal	2.43 ± 1.10	-58.20 ± 10.76	-0.27 ± 0.03
		DMD	-0.74 ± 0.87	-40.64 ± 5.64	-0.29 ± 0.03
	Contraction				
		Normal	0.67 ± 0.75	-34.85 ± 8.97[a]	-0.16 ± 0.03[a]
		DMD	0.23 ± 0.77	-36.56 ± 4.85	-0.27 ± 0.03
After 7-NI					
	Rest				
		Normal	0.39 ± 4.05	-51.43 ± 9.76	-0.24 ± 0.02
		DMD	0.02 ± 1.22	-39.83 ± 4.27	-0.32 ± 0.03
	Contraction				
		Normal	1.07 ± 1.54	-48.71 ± 8.48	-0.24 ± 0.03
		DMD	-0.51 ± 1.47	-36.70 ± 4.49	-0.29 ± 0.03
After L-NAME					
	Rest				
		Normal	0.52 ± 1.13	-68.32 ± 9.73	-0.27 ± 0.03
		DMD	1.15 ± 1.20	-46.56 ± 5.29	-0.30 ± 0.03
	Contraction				
		Normal	0.87 ± 0.95	-67.17 ± 12.47[b]	-0.26 ± 0.03
		DMD	0.35 ± 1.16	-43.66 ± 5.47	-0.27 ± 0.03

Abbreviations: MAP, mean arterial pressure; MABF, mean arterial blood flow; MVC, mean vascular conductance; DMD, Duchenne muscular dystrophy.

a, Significantly different from that of normal at rest.

b, Significantly different from that of normal at baseline and after 7-NI.

Table 3. Hemodynamic responses to NE induced sympathetic vasoconstriction

Drug condition	Muscle contractile status	Group	Before NE			After NE		
			MAP (Hgmm)	MABF (mL/min)	MVC (mL/min/Hgmm/100	MAP (Hgmm)	MABF (mL/min)	MVC (mL/min/Hgmm/100
Baseline								
	Rest							
		Normal	66.76 ± 3.86	99.87 ± 19.00	0.46 ± 0.05	69.19 ± 3.28	41.67 ± 13.00[a]	0.19 ± 0.05[a]
		DMD	72.14 ± 4.21	52.60 ± 7.14[b]	0.38 ± 0.04	71.40 ± 3.75	11.96 ± 2.12[a,b]	0.08 ± 0.01[a,b]
	Contraction							
		Normal	67.17 ± 4.86	123.18 ± 26.55	0.54 ± 0.07	67.84 ± 4.60	88.33 ± 21.72[a]	0.37 ± 0.06[a]
		DMD	71.66 ± 4.04	63.99 ± 7.05[b]	0.48 ± 0.05	71.89 ± 3.90	27.44 ± 3.51[a,b]	0.21 ± 0.03[a,b]
After 7-NI								
	Rest							
		Normal	66.19 ± 4.66	95.76 ± 16.44	0.46 ± 0.05	66.58 ± 4.05	44.32 ± 10.79[a]	0.22 ± 0.05[a]
		DMD	64.96 ± 3.33	48.66 ± 5.13[b]	0.40 ± 0.04	64.99 ± 3.30	8.84 ± 1.41[a,b]	0.08 ± 0.01[a,b]
	Contraction							
		Normal	64.46 ± 3.28	119.83 ± 20.49	0.58 ± 0.06	65.52 ± 3.85	71.12 ± 13.65[a]	0.34 ± 0.05[a]
		DMD	64.93 ± 3.48	59.29 ± 5.77[b]	0.50 ± 0.05	64.42 ± 3.27	22.59 ± 2.76[a,b]	0.20 ± 0.04[a,b]
After L-NAME								
	Rest							
		Normal	83.74 ± 3.31	126.98 ± 16.82	0.50 ± 0.06	84.26 ± 3.86	58.66 ± 12.10[a]	0.23 ± 0.05[a]
		DMD	80.26 ± 4.17	59.99 ± 5.89[b]	0.40 ± 0.04	81.41 ± 3.69	13.43 ± 1.75[a,b]	0.10 ± 0.02[a,b]
	Contraction							
		Normal	81.57 ± 3.72	158.43 ± 24.15	0.61 ± 0.08	82.43 ± 3.50	91.27 ± 15.04[a]	0.35 ± 0.06[a]
		DMD	83.05 ± 3.75	73.13 ± 6.10[b]	0.48 ± 0.05	83.40 ± 3.60	29.47 ± 2.90[a,b]	0.21 ± 0.04[a,b]

Abbreviations: NE, norepinephrine; MAP, mean arterial pressure; MABF, mean arterial blood flow; MVC, mean vascular conductance; DMD, Duchenne muscular dystrophy.

a, Significantly different from that of before NE.

b, Significantly different from that of normal.

***Additional accomplishments that have benefited from this grant*.**

Accomplishment benefited from this grant 1. In this study, we have proposed to use intravenous injection to deliver AAV micro-dystrophin vectors to affected dogs. We published a review article on the current state-of-art on systemic AAV delivery in animal models (Duan, 2016).

Accomplishment benefited from this grant 2. The ultimate goal of this project is to develop the best micro-dystrophin AAV gene therapy. A better understanding of dystrophin biology is essential to decide which part(s) of the dystrophin coding sequence should be included in the synthetic micro-dystrophin gene. To this end, we identified 3 new membrane-binding domains in full-length dystrophin. This information will be used to engineer future more functional micro-dystrophin genes (Zhao et al 2016).

Accomplishment benefited from this grant 3. A major concern of AAV micro-dystrophin therapy is that the therapeutic microgene may lose over time. A previous study from Dickson lab suggests that such loss may not compromise therapeutic benefits. In other words, there is no need for re-administration. We now took a more stringent genomic approach and revisited this question. Our results suggest that s loss of therapeutic mini-dystrophin is accompanied with the loss of protection in both skeletal muscle and the heart. Our study corrected an important misconception in the field of AAV dystrophin replacement therapy (Wasala et al 2016).

Accomplishment benefited from this grant 4. In our initial study on systemic AAV micro-dystrophin delivery in young adult dystrophic dogs (these data were presented in previous progress

reports), we found robust transduction in skeletal muscle but gene transfer in the heart is limited with AAV-9. To address whether low-level dystrophin expression in the heart can still offer some protection, we studied mdx3cv mice which expressed ~3.8% of dystrophin in the heart. We found that this low-level dystrophin expression resulted in slight, but significantly better functional rescue in aged mice than mdx4cv mice which have no dystrophin in the heart. This result suggests that some dystrophin is better than no dystrophin. It support our continued pursuing of AAV micro-dystrophin therapy (Wasala et al 2017)

Accomplishment benefited from this grant 5. In this study, we have proposed to use AAV vectors as the delivery tool. AAV is a bio-nanoparticle. We published a review article on the current state-of-art on nanotherapy (both viral and noviral) for DMD (Nance et al, 2017).

Accomplishment benefited from this grant 6. Repeated biopsy is a burden to patients following AAV micro-dystrophin gene therapy. Electrical impedance myography is a recently developed technology that will allow investigators to study muscle architecture without performing biopsy. In this funding period, we established the protocol for using electrical impedance myography to evaluate normal and dystrophic dog muscles. We have now published this work (Hakim et al 2017 PLoS One).

Accomplishment benefited from this grant 7. In the last progress report, we showed the data on the evaluation of the latest muscle-specific CK8 promoter in a severely affected mouse model. We have now published this work (Hakim et al 2017 Molecular Therapy Methods and Clinical Development).

Training and professional development opportunities. Nothing to report.

Dissemination of the results. Above mentioned studies and review articles have been either published in peer-reviewed scientific journals or presented in academic conferences.

Plan for future.

With the support from the DoD MD130014 grant, we have successfully demonstrated persistent (up to 2 years) systemic AAV micro-dystrophin therapy in young adult affected dogs. Treatment ameliorated muscle histopathology and improved muscle force in affected dogs. No untoward side reactions were observed in dogs that have received systemic AAV micro-dystrophin therapy. Partially because of our data, several human trials have been planned in the United State and Europe. These include Solid Biosciences trial (https://solidbio.com/content/advancing-toward-clinic), Nationwide Children's Hospital/Sarepta/PPMD trial (https://musculardystrophynews.com/2017/09/14/awaited-duchenne-md-gene-therapy-trial-topic-of-ppmd-webinar-with-jerry-mendell-nationwide-childrens-hospital/), Pfizer/Bamboo trial (www.pfizer.com/news/press-release/press-release-detail/pfizer_aims_to_become_industry_leader_in_gene_therapy_with_aquisition_of_bamboo_therapeutics_inc) and Genethon trial (www.dddmag.com/article/2017/06/sarepta-signs-gene-therapy-r-d-deal-dmd).

With the support from the DoD MD130014 grant, we have also engineered a new version of the next generation AAV micro-dystrophin vector (XP49, see Figure 3). We have administrated this vector to six young adult affected dogs. We will continually monitor and study these dogs. Since this

new vector has a number of features that are missing in the current generation vectors, we expect results from these dogs will lay the foundation to the development of more efficacious AAV micro-dystrophin gene therapy in human patients in the future.

With the support from the DoD MD130014 grant, we have also developed a number of novel physiological assays to quantitatively evaluate dystrophic phenotype in affected dogs. These assays include (1) non-invasive evaluation of dog activity (Hakim CH, Peters AA, Feng F, Yao G, **Duan D.** *Night activity reduction is a signature physiological biomarker for Duchenne muscular dystrophy dogs.* **Journal of Neuromuscular Diseases**. 2(4):397-407, **2015**); (2) non-invasive evaluation of dog muscle architecture using electrical impedance myography (Hakim CH, Mijailovic A, Lessa TB, Coates JR, Rutkove SB, **Duan D.** *Non-invasive evaluation of muscle disease in the canine model of Duchenne muscular dystrophy by electrical impedance myography*. **PLoS One** 12(3):e0173557**, 2017**); (3) quantitative evaluation of dog muscle blood perfusion and functional ischemia **(Figures 4 to 6, Tables 1 to 3)**.

It is worth to point out that establishment of a set of robust outcome measurements is essential for the success of preclinical studies. In the absence of rigorous assays, it will be impossible to reach a solid conclusion to guide human trials. The assays we developed will not only benefit AAV micro-dystrophin gene therapy studies in the canine DMD model, they will also benefit any future experimental therapeutic studies in the canine model such as CRISPR-mediated gene editing, stem cell therapy, dystrophin-independent disease modulating gene therapy, and drug therapy.

In light of the rapid progress in this field, we expect that there will be a huge demand for the AAV micro-dystrophin vector to meet the needs of human patients. The traditional AAV production method is transient transfection in 293 cells. This method is labor intensive and costly. Importantly, it cannot be scaled up. Scalable AAV production methods have been published. However, it is unclear whether the quality of the AAV vector generated using the scalable methods can reach that of the transient transfection method. As a preparation for future AAV micro-dystrophin gene therapy in human patients, we opt to address this important issue during the no-cost extension period. Specifically, we will compare the in vivo performance of AAV vectors made by either transient transfection or a scalable method side-by-side. We will quantify the vector genome, level of micro-dystrophin expression (by immunostaining and western blot), histological and physiological rescue in a mouse DMD model.

4. Impact. Nothing to report.

5. Changes/Problems.

Nothing to report.

6. Products

6.1. Peer-reviewed publications (a total of 7) (All 7 publications have federal support)

1) **Duan D.** *Systemic delivery of adeno-associated viral vectors.* **Current Opinion in Virology** 21:16-25, **2016**
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions,

and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

2) Zhao J, Kodippili K, Yue Y, Hakim CH, Wasala L, Pan X, Zhang K, Yang NN, **Duan D**, Lai Y. *Dystrophin contains multiple independent membrane-binding domains.* **Human Molecular Genetics** 25(10):3647-3653**, 2016** (DD and YL as co-corresponding authors).
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

3) Wasala NB, Lai Y, Shin J-H, Zhao J, Yue Y, **Duan D.** *Genomic removal of a therapeutic mini-dystrophin gene from adult mice elicits a Duchenne muscular dystrophy-like phenotype.* **Human Molecular Genetics** 25(13):2633-2644**, 2016**
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

4) Wasala NB, Yue Y, Jenna Vance, **Duan D.** *Uniform low-level dystrophin expression in the heart partially preserved cardiac function in an aged mouse model of Duchenne cardiomyopathy.* **Journal of Molecular and Cellular Cardiology** 102:45-52, **2017**
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

5) Nance ME, Hakim CH, Yang NN and **Duan D.** *Nanotherapy for Duchenne muscular dystrophy.* **WIREs Nanomedicine and Nanobiotechnology** e1472**, 2017**
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

6) Hakim CH, Mijailovic A, Lessa TB, Coates JR, Rutkove SB, **Duan D.** *Non-invasive evaluation of muscle disease in the canine model of Duchenne muscular dystrophy by electrical impedance myography.* **PLoS One** 12(3):e0173557**, 2017**
"This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

7) Hakim CH, Wasala NB, Pan X, Kodippili K, Yue Y, Zhang K, Yao G, Haffner B, Duan XS, Schneider JS, Yang NN, Chamberlain JS, **Duan D.** *A five-repeat micro-dystrophin gene ameliorated dystrophic phenotype in the severe DBA/2J-mdx model of Duchenne muscular dystrophy.* **Molecular Therapy-Methods & Clinical Development** 6:216-230**, 2017** "This work was supported in part by the Department of Defense, Duchenne Muscular Dystrophy Research Program (DMDRP), Congressionally Directed Medical Research Programs under Award No. W81XWH-14-1-0302." "Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the Department of Defense."

6.3. Conference presentations (a total of 8)

1) Angus Lindsay, Dawn A. Lowe, **Dongsheng Duan**, Luke M. Judge, Jeffery S. Chamberlain and James M. Ervasti. *Deletion of sequences encoding spectrin repeat 2 through hinge 2 from micro-dystrophin compromises protection from eccentric contraction-induced force drop in mdx mice.* Advances in Skeletal Muscle Biology in Health and Disease. University of Florida, Gainesville, FL. March 8-10, 2017

2) Chady H. Hakim, Kasun Kodippili, Gregory Jenkins, Hsiao T. Yang, Xiufang Pan, Thais B. Lessa , Stacey B. Leach, Craig Emter, Yongping Yue, Keqing Zhang, Sean X. Duan, Gang Yao, Joel S. Schneider, Nora N. Yang, Jeffrey S. Chamberlain, **Dongsheng Duan**. *Single systemic AAV micro-dystrophin therapy ameliorates muscular dystrophy in young adult Duchenne muscular dystrophy dogs for up to two years.* 2017 Inaugural Musculoskeletal Regenerative Medicine and Biology Meeting. Saint louis, MO. May 4-6, 2017. **(selected for oral presentation)**

3) Chady H. Hakim, Kasun Kodippili, Gregory Jenkins, Hsiao T. Yang, Xiufang Pan, Thais B. Lessa , Stacey B. Leach, Craig Emter, Yongping Yue, Keqing Zhang, Sean X. Duan, Gang Yao, Joel S. Schneider, Nora N. Yang, Jeffrey S. Chamberlain, **Dongsheng Duan**. *Single systemic AAV micro-dystrophin therapy ameliorates muscular dystrophy in young adult Duchenne muscular dystrophy dogs for up to two years.* 2017 20th Annual Meeting of the American Society of Gene & Cell Therapy. Washington, DC May 10-13, 2017

4) Chady H. Hakim, Kasun Kodippili, Gregory Jenkins, Hsiao T. Yang, Xiufang Pan, Thais B. Lessa , Stacey B. Leach, Craig Emter, Yongping Yue, Keqing Zhang, Sean X. Duan, Gang Yao, Joel S. Schneider, Nora N. Yang, Jeffrey S. Chamberlain, **Dongsheng Duan.** *Single systemic AAV micro-dystrophin therapy ameliorates muscular dystrophy in young adult Duchenne muscular dystrophy dogs for up to two years.* 2017 4th Ottawa International Conference on Neuromuscular Disease and Biology. Ottawa, Ontario, Canada Sep 7-9, 2017

5) Hakim CH, Wasala NB, Pan X, Kodippili K, Yue Y, Zhang K, Yao G, Haffner B, Duan XS, Schneider JS, Yang NN, Chamberlain JS, **Dongsheng Duan**. *A five-repeat micro-dystrophin gene ameliorated dystrophic phenotype in the severe DBA/2J-mdx model of Duchenne muscular dystrophy.* 2017 4th Ottawa International Conference on Neuromuscular Disease and Biology. Ottawa, Ontario, Canada Sep 7-9, 2017

6) Chady H. Hakim, Nathalie Clement, Lakmini P. Wasala, Hsiao T. Yang, Yongping Yue, Keqing Zhang, Kasun Kodippili, Joel S. Schneider, Nora N. Yang, Jeffrey S. Chamberlain, Barry J. Byrne, **Dongsheng Duan** *In vivo comparison of the biological potency of rAAV9-microdystrophin made by transient transfection and a scalable herpesvirus system* 2017 4th Ottawa International Conference on Neuromuscular Disease and Biology. Ottawa, Ontario, Canada Sep 7-9, 2017

7) Nalinda B. Wasala, Jinhong Shin, Yi Lai, Yongping Yue, Federica Montanaro, **Dongsheng Duan**. *R16-19 is a putative heart protection domain in dystrophin* 2017 4th Ottawa International Conference on Neuromuscular Disease and Biology. Ottawa, Ontario, Canada Sep 7-9, 2017

8) D. M. Nelson, **Dongsheng Duan**, L. M. Judge, J. S. Chamberlain and J.M. Ervasti *Variable rescue of microtubule defects in mdx skeletal muscle expressing miniaturized dystrophins* 2017 ASCB-EMBO Annual Meeting. Philadelphia, PA, Dec 2-6, 2017.

7. Participants/collaborating organizations:

What individuals have worked on the project?

Name: Dongsheng Duan – "No change"

Name: Craig Emter – "No change"

Name: Yi Lai – "No change"

Name: Hsiao Tung "Steve" Yang – "No change"

Name: Yongping Yue – "No change"

Name: Aihua Dai
Project Role: Research Specialist
Research Identifier: not applicable
Nearest person month worked: 1
Contribution to the Project: Quantifying viral genome copy number in muscle samples collected from dogs.

Changes in the active other support of the PI and key personnel since the last reporting period.

Dongsheng Duan, PI

Previous/active grants that have closed:
<u>Maximum feasible dose study in a canine model of Duchenne muscular dystrophy</u>
10% effort, Duan, PI
Solid GT, LLC
07/01/2016-04/30/2017
$80,000 (direct costs)

This study will assess the expression, localization and bio-distribution of canine SGT-001.

Current research support:

Whole body single AAV microgene therapy in canine DMD
16% effort, PI
NIH, NINDS (R01 NS090634)
09/01/2015-07/31/2020
$406,687 (direct costs/current year)
In this study, we will test whether a newly developed canine Y731F AAV-9 micro-dystrophin vector gene therapy can lead to clinically meaningful improvement in dystrophic dogs.
Specific aim 1 is to test regional therapy in the hope of applying it to improve life quality in late-stage patients and aim 2 is to test systemic therapy in the hope of achieving bodywide improvement in young patients.
(There is no scientific/budget overlap with the current proposal).

R16/17-independent nNOS anchoring mechanism
8% effort, PI
NIH/NIAMS (R21 AR067985)
04/01/2016-03/31/2018
$110,000 (direct costs/current year)
The goal is to identify the dog nNOS-binding domain and develop relevant gene delivery vectors.
The specific aims are (1) to identify the canine specific nNOS-binding domain in dog dystrophin and (2) to develop the nNOS-binding canine dystrophin adeno-associated virus (AAV) vector.
(There is no scientific/budget overlap with the current proposal.)

CRISPR/Cas9-based gene editing for the correction of Duchenne muscular dystrophy
8% effort, Co-PI (PI: Charles Gersbach)
Duke University, NIH (R01 AR069085)
04/01/2016-03/31/2021
$70,691 (direct costs/current year)
The Duan lab will perform in vivo gene delivery and functional outcome measurements in mice treated by AAV-CRISPR gene repair vectors and if needed will also assist with the production of recombinant AAV vectors.
Specific aim: To test CRSPR/Cas9 gene therapy to treat muscle disease in mdx mice and hDMD mice.
(There is no scientific/budget overlap with the current proposal)

A pilot study to evaluate long-term safety and efficacy of AAV-9 5Rc micro-dystrophin therapy
4% effort, PI
Solid Biosciences
06/01/2016-05/31/2019
$100,000 (direct costs/current year)
The overarching goal of this project is to determine whether systemic AAV-9 5Rc micro-dystrophin gene therapy can yield long-term (up to 4 years after injection) microgene expression without causing serious adverse events (SAEs).
(There is no scientific/budget overlap with the current proposal.)

Treatment of Duchenne muscular dystrophy with the muscle calcium pump
16% effort, PI
NIH/NIAMS (R01 AR070517)
07/01/2016-08/31/2021
$406,296 (direct costs/current year)
Goal: Elevation of cytosolic calcium is a pivotal pathogenic event in Duchenne muscular dystrophy (DMD). We found that sarco/endoplasmic reticulum calcium ATPase 2a (SERCA2a) therapy can reduce muscle disease and improve muscle function in the mouse DMD model. In the proposed study, we will test whether this therapy can treat symptomatic DMD dogs and our results will lay the foundation for a future clinical trial.
The specific aims are: (1) to test whether regional AAV SERCA2a therapy can ameliorate limb muscle disease and improve function and (2) to test whether systemic AAV SERCA2a therapy can lead to bodywide improvement in affected dogs.
(There is no scientific/budget overlap with the current proposal.)

Evaluation of the human version second-generation AAV micro-dystrophin vector in adult dystrophic dogs
3% effort, PI
Jesse's Journey; The Foundation for Gene & Cell Therapy
7/1/14-12/31/2017 (extension)
$100,000 CAN (direct costs/year)
Goal: We propose to generate the human version microgene vector and confirm its function in adult DMD dogs.
The specific aims are: (1) to engineer a codon-optimized second-generation human dystrophin microgene in a customer-optimized expression cassette and package it in an AAV-8 vector; (2) to validate the efficacy of the human version vector in dystrophin deficient mdx mice by systemic gene transfer; (3) to validate the efficacy of the human version vector in adult dystrophic dogs by local gene transfer; (4) to explore systemic gene therapy in young adult dystrophic dogs.
(This is a supplementary grant to the DOD grant awarded September 2014 that has been approved for extension to 12/31/2017).
(There is no scientific/budget overlap with the current proposal.)

Treating Duchenne cardiomyopathy in the mouse model by gene repair
8% effort, Duan, PI
Department of Defense W81XWH-16-1-0221
08/01/2016-07/31/2019
$191,667 (direct costs)
We propose to test this "permanent exon skipping" therapy to the treatment of Duchenne cardiomyopathy in an authentic mouse model. Our study will open the door to the eventual application of CRISPR/Cas9 therapy in human patients in the future.
(There is no scientific/budget overlap with the current proposal.)

New/active grants:
Fine-needle microscopic tractography for in vivo high-resolution imaging of muscle damage
2% effort, Co-PI (PI: Gang Yao)
University of Missouri, Interdisciplinary Pilot Studies in Translational Science and Biomedical

Innovations
07/01/2017 to 06/30/2018
$50,000 (direct costs/current year)
The goal and aim of this project is to develop a new microscopic imaging method for minimal invasive imaging of muscle damage.
(There is no scientific/budget overlap with the current proposal.)

Evaluation of Montelukast as a potential therapy for Duchenne muscular dystrophy in the murine model
3% effort, PI
Duchenne UK
03/01/2017-02/28/2020
$60,000 (direct costs/current year)
We propose to evaluate safety and therapeutic efficacy of Montelukast in mdx mice, the most commonly used mouse model for DMD.
(There is no scientific/budget overlap with the current proposal)

Evaluation of Montelukast as a potential therapy for Duchenne muscular dystrophy in the murine model
0% effort, PI
Michael's Cause
03/01/2017-02/28/2020
$16,667 (direct costs/current year)
We propose to evaluate safety and therapeutic efficacy of Montelukast in mdx mice, the most commonly used mouse model for DMD. This is a supplementary grant to the Duchenne UK grant.
(There is no scientific/budget overlap with the current proposal)

Evaluation of Montelukast as a potential therapy for Duchenne muscular dystrophy in the murine model
0% effort, PI
Ryan's Quest
03/01/2017-02/28/2020
$16,667 (direct costs/current year)
We propose to evaluate safety and therapeutic efficacy of Montelukast in mdx mice, the most commonly used mouse model for DMD. This is a supplementary grant to the Duchenne UK grant.
(There is no scientific/budget overlap with the current proposal)

Evaluation of Montelukast as a potential therapy for Duchenne muscular dystrophy in the murine model
0% effort, PI
Rally for Ryan
03/01/2017-02/28/2020
$16,667 (direct costs/current year)
We propose to evaluate safety and therapeutic efficacy of Montelukast in mdx mice, the most commonly used mouse model for DMD. This is a supplementary grant to the Duchenne UK grant.
(There is no scientific/budget overlap with the current proposal)

DMD gene therapy in the canine model by intramuscular sarcolipin knockdown
5% effort, PI
Jesse's Journey: The Foundation for Gene & Cell Therapy
08/01/2017-07/31/2020
$131,515 CAN/year
The major goal of this study is to demonstrate that sarcolipin (SLN) knockdown improves the carco/endoplasmic reticulum calcium ATPase (SERCA) function and ameliorate the muscle disease in a dog model of Duchenne muscular dystrophy.
(There is no scientific/budget overlap with the current proposal)

Pilot study to evaluate protein CRISPR therapy in the DMD mouse model
0% effort, PI
Hubrecht Institute – Utrecht University, Netherlands
10/01/2017-09/30/2019
$10,000 (direct costs/year)
In this pilot study, the Geijsen lab and the Duan lab will analyze the functional improvement of a mouse model of Duchenne muscular dystrophy upon Dystrophin gene repair by the iTOP-mediated introduction of Cas9 and sgRNA to skeletal muscle fibers and satellite cells of (DMD) to test DMD CRISPR therapy.
(There is no scientific/budget overlap with the current proposal.)

Evaluation of osteoprotegerin (OPG) in the mdx model of Duchenne muscular dystrophy
7% effort, PI
Ryan's Quest
10/15/2017-04/15/2018
$83,385 (direct costs)
To validate muscle protection effect of osteoprotegerin (OPG) in 25 day-old mdx mice.
(There is no scientific/budget overlap with the current proposal.)

Craig Emter, Co-PI

Previous/active grants that have closed:
Translational swine model for the study of HFpEF
10% effort, Emter, PI
University of Missouri Research Board
01/01/2015-12/31/2016
$45,000 total
Major goals: Pilot clinical and translational studies for developing an obese and diabetic ossabaw swine model of HFpEF.

Regulation of Work Capacity in Cardiac Myocytes
McDonald, PI
5% effort, Emter, Co-I
NIH/NHLBI, R01 HL57852-14
3/1/12-3/1/2017
$250,000 (direct costs/year)

Major Goals: The goal of this project is to investigate the determinants of power output in myocardium.

Pathological mechanisms of sympathetic-mediated cerebrovascular vasoconstriction in heart failure with preserved ejection fraction
5% effort, Emter/Olver, Co-PI's
University of Missouri, Internal
College of Veterinary Medicine COR Faculty Research Program
1/1/16-12/31/16
$18,000 total funding
Major goals: Pilot clinical and translational studies for examining sympathetic nervous system contributions to developing heart failure in a mini-swine model of HFpEF
Role: PI

New/active:
Coronary Dysfunction, BK Channels, & Exercise in Heart Failure
33% effort, Emter, PI
NIH/NHLBI, R01 HL112998
5/1/14-4/30/2019
$250,000 (direct costs/year)
Major Goals: The goal of this project is to determine the role of the coronary vascular BK_{Ca} channel in the development of heart failure with preserved ejection fraction.
(There is no scientific/budget overlap with the current proposal.)

Mechanisms of sympathetic-mediated cerebrovascular vasoconstriction in heart failure with preserved ejection fraction
AHA Postdoctoral Fellowship (Olver, PI)
0% effort, Emter, Supervising PI
American Heart Association
1/1/2016-12/31/2017
$50,000 total funding
Major goals: Salary support for research career development.
(There is no scientific/budget overlap with the current proposal.)

Pathological Mechanisms of Sympathetic-mediated Cerebrovascular Vasoconstriction as a Function of Menopause in Heart Failure with Preserved Ejection Fraction
Olver, PI
0% effort, Emter, Supervising PI
University of Missouri, Internal
MU Interdisciplinary Center on Aging - Research Enrichment and Dissemination (READ) Small Grants Program
1/22/16-12/31/18
$10,000 (direct costs/year)
Major goals: Pilot clinical and translational studies for examining sympathetic nervous system contributions to developing heart failure in a mini-swine model of HFpEF
(There is no scientific/budget overlap with the current proposal.)

Yi Lai, Associate Research Professor

Previous/active grants that have closed:
Over-expressing nNOS as a therapy for DMD cardiac disease
50% effort, Lai, PI
Duchenne Parent Project (The Netherlands)
04/01/2015-05/31/2017
€200,000 total budget
This project will reveal therapeutic mechanisms of deltaPDZ nNOS expression and promote the development of AAV.deltaPDZ nNOS therapy for DMD cardiac disease. Given the therapeutic potential of nNOS over-expression in multiple cardiac diseases, this project will also benefit the treatment of other cardiac diseases.
(There is no scientific/budget overlap with the current proposal.)

Current research support:
R16/17-independent nNOS anchoring mechanism
10% effort, Co-I (Dongsheng Duan, PI)
NIH/NIAMS (R21 AR067985-01A1)
04/01/2016-03/31/2018
$110,000 (direct costs/current year)
The goal is to identify the dog nNOS-binding domain and develop relevant gene delivery vectors. The specific aims are (1) to identify the canine specific nNOS-binding domain in dog dystrophin and (2) to develop the nNOS-binding canine dystrophin adeno-associated virus (AAV) vector.
(There is no scientific/budget overlap with the current proposal.)

New/active grants:
None

Hsiao Tung Yang, Research Professor

Previous/active grants that have closed:
Solid GT Fellowship Award
62% effort, Yang, PI
Solid GT
05/01/2016-07/31/2017
$100,921 total funding
The goal is to support the position of Dr. Hsiao-Tung Yang, assisting Dr. Dongsheng Duan in his work helping Solid GT's efforts in gene therapy for Duchenne Muscular Dystrophy. Dr. Yang will be responsible for program management, program execution and study report delivery.
(There is no scientific/budget overlap with the current proposal.)

Current research support:
None

New/active grants:
None

8. Special reporting reqirements: None

9. Appendices:

Peer-reviewed publications